Treatment of thromboangiitis obliterans (Buerger's disease) by intramuscular gene transfer of vascular endothelial growth factor: Preliminary clinical results

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Purpose: **Thromboangiitis obliterans (TAO), or Buerger's disease, a distinct form of vascular occlusive disease that afflicts the peripheral arteries of young smokers, is often characterized by an inexorable downhill course even in patients who discontinue smoking once a stage of critical limb ischemia associated with ulceration or gangrene is reached. As part of a phase I clinical trial to document the safety and efficacy of intramuscular gene transfer of naked plasmid DNA-encoding vascular endothelial growth factor ($phVEGF_{165}$) in the treatment of critical limb ischemia, we treated TAO in 6 patients.**

Methods: **Seven limbs in 6 patients (3 men, 3 women; mean age, 33 years; range, 33 to 51 years) who satisfied the criteria for TAO and had signs or symptoms of critical limb ischemia were treated twice, 4 weeks apart, with 2 or 4 mg of $phVEGF_{165}$, which was administered by direct intramuscular injection at 4 arbitrarily selected sites in the ischemic limb. The gene expression was documented by enzyme-linked immunosorbent assay that was performed on peripheral blood samples.**

Results: **The ulcers that were nonhealing for more than 1 month healed completely in 3 of 5 limbs after the intramuscular $phVEGF_{165}$ gene therapy. Nocturnal rest pain was relieved in the remaining 2 patients, although both continue to have claudication. The evidence of the improved perfusion to the distal ischemic limb included an increase of more than 0.1 in the ankle brachial index in 3 limbs, an improved flow shown with magnetic resonance imaging in 7 of the 7 limbs, and newly visible collateral vessels shown with serial contrast angiography in 7 of the 7 limbs. The adverse consequences of the $phVEGF_{165}$ gene tranfer were limited to transient ankle or calf edema in 3 of the 7 limbs. Two patients with advanced distal forefoot gangrene ultimately required below-knee amputation despite the evidence of improved perfusion. A histologic section disclosed the classic pathologic findings of TAO.**

Conclusion: **Therapeutic angiogenesis with $phVEGF_{165}$ gene transfer, if instituted before the development of forefoot gangrene, may provide a novel therapy for patients with advanced Buerger's disease that is unresponsive to standard medical or surgical treatment methods. (J Vasc Surg 1998;28:964-75.)**

Buerger's disease, which is also referred to by its pathologic description of thromboangiitis obliterans (TAO), is now widely recognized as a specific disease entity that is characterized by the onset of distal extremity ischemic symptoms at an early age in the absence of an underlying proximal embolic source, trauma, autoimmune disease, diabetes, or hyperlipidemia.[1] Although Buerger's disease is worldwide in its distribution, it is far more prevalent in the middle, near, and far east regions than in North America.[2] It occurs predominantly in men, but recent evidence indicates that the incidence rate in women is increasing.[3] Virtually all of the patients are heavy users of tobacco, usually cigarettes. The disease generally is seen first

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with claudication (often at the foot level) but, if cigarette smoking continues, often progresses to critical limb ischemia with dependent rubor, trophic changes, and ultimately digital gangrene or gangrenous ulcers.

Clinical experience has shown that if the disease is diagnosed before the onset of gangrene or tissue loss, then patients with Buerger's disease can be assured that the complete cessation of tobacco use will generally result in a benign course with the avoidance of future amputation.[2] On the other hand, all other forms of therapy, including the use of prostaglandins, anticoagulant or thrombolytic therapy, and surgical reconstruction, are palliative and often unsuccessful in the setting of continued smoking. Surgical revascularization, in particular, does not usually produce a durable result because of the diffuse distal nature of the obstructing lesions in Buerger's disease. Most patients, in fact, are not candidates for reconstruction because a distal target vessel for bypass grafting cannot be identified. Inada et al,[4] for example, found that only 4.6% of 236 patients could be treated by surgical means.

Preclinical studies have shown that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia—a concept now referred to as therapeutic angiogenesis.[5-8] Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a secreted endothelial cell–specific mitogen that has been shown to be particularly effective in these studies because of its high affinity binding to endothelial cells (the most critical cellular element responsible for new vessel formation).[6] For the past 18 months, we have been conducting a phase I clinical trial to document the safety and efficacy of the use of intramuscular gene transfer of naked plasmid DNA-encoding for the 165 amino acid isoform of VEGF (phVEFG$_{165}$) in patients with critical limb ischemia. As part of that study, 6 patients (7 limbs) who met the criteria for TAO have undergone treatment, and this forms the basis of this report.

METHODS

Patients. Six patients of the first 22 who enrolled in a phase I nonrandomized study to document the safety of intramuscular phVEGF$_{165}$ gene transfer met the following diagnostic criteria for Buerger's disease: the onset of distal extremity ischemic symptoms before the age of 50 years; the absence of an underlying proximal embolic source, trauma, autoimmune disease, diabetes, or hyperlipidemia; healthy arteries proximal to the distal superficial femoral artery segment; and distal occlusive disease with distinctive arteriographic or pathologic findings.

The patients were considered to be candidates for intramuscular gene therapy if they met the following criteria: (1) the patients had chronic critical limb ischemia, including rest pain or nonhealing ischemic ulcers, that was present for a minimum of 4 weeks without evidence of improvement in response to conventional therapy; and (2) the patients were not candidates for either surgical or percutaneous revascularization procedures. In addition, objective documentation of ischemia, including a resting ankle brachial index (ABI) of less than 0.6 or a toe brachial index (TBI) of less than 0.5, in the affected limb on 2 consecutive examinations performed 1 week apart was necessary. The criteria that were used to document a change in limb status were adopted from the standards recommended by The Society of Vascular Surgery/North America Chapter of the International Society of Cardiovascular Surgery. This study was a phase I nonrandomized trial designed to document the safety of intramuscular phVEGF$_{165}$ gene transfer and to monitor patients for evidence of bioactivity. The design was approved by the US Food and Drug Administration, the Recombinant DNA Advisory Committee of The National Institutes of Health, and the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth's Medical Center.

Plasmid DNA (phVEGF$_{165}$). Each patient received an intramuscular injection of a eukaryotic expression vector that encoded the VEGF$_{165}$ gene whose transcription was regulated by cytomegalovirus promoter/enhancer. The preparation and purification of the plasmid from cultures of phVEGF$_{165}$-transformed Escherichia coli was performed in the human gene therapy laboratory at St Elizabeth's Medical Center with the column method (Qiagen Mega Kit, Qiagen, Inc, Valencia, Calif). The purified plasmid was stored in vials and pooled for quality-controlled analyses.

Intramuscular phVEGF$_{165}$ gene transfer. Aliquots of 500 μg of VEGF$_{165}$ plasmid DNA were diluted in a sterile saline solution, and 4 aliquots (total of 2000 μg) were administered into the calf or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were selected arbitrarily according to the available muscle mass and included sites above and below the knee. The volume of each of the 4 injections per limb progressively was increased during the course of the study from 0.5 mL to 3 mL to 5 mL. Four weeks after the first 2000-μg injection, a second 2000-μg injection was administered for a total of 4000 μg of plasmid DNA per patient.

Patient follow-up and assessment. The patients

Table I. Clinical features of 6 patients (7 limbs)

Patient characteristics					*Clinical findings*		*DSA/MRA*	
No.	*Age (years)*	*Sex*	*Previous treatment*	*Follow-up (months)*	*Before Gtx*	*After Gtx*	*Before Gtx*	*After Gtx*
1	33	F	4 bypass grafts (occluded) and prostaglandin therapy	18	toe gangrene	healing of gangrene	SFA occlusion	plus new collaterals
2	39	F	sympathectomy	17	forefoot gangrene	BKA	SFA, POP occlusion	plus new collaterals
3	39	F	sympathectomy	14	toe gangrene, heel ulcer	toe amputation, limb salvage	AT, PT, PN occlusion	plus new collaterals
4	44	M	3 bypass grafts (occluded) and prostaglandin therapy	12	forefoot gangrene	BKA	SFA, POP occlusion	plus new collaterals
5	51	M	narcotic analgesics	12	rest pain	walking of 5 minutes without pain	SFA occlusion	plus new collaterals
6	26	M	prostaglandin therapy	7	toe gangrene	healing of gangrene	AT, PT, PN occlusion	plus new collaterals
7	46	M	none	3	toe gangrene	improvement of gangrene	AT, PT, PN occlusion	plus new collaterals

DSA, Digital subtraction angiography; *MRA,* magnetic resonance angiography; *Gtx,* gene therapy; *SFA,* superficial femoral artery; *BKA,* below-knee amputation; *POP,* popliteal; *AT,* anteriortibial; *PT,* posterior tibial; *PN,* peroneal.

were followed weekly on an outpatient basis during the first 8 weeks after the initial gene therapy procedure and then at monthly intervals thereafter. Ischemic ulcers were documented with color photography. The patients received analgesics as necessary for the management of rest pain, and their requirements were carefully documented. Ischemic ulcers and necrotic lesions were managed in a standard fashion, and antibiotics were prescribed as necessary. No special forms of therapy or bed rest were used during the study period. The resting ankle brachial index and the toe brachial index were calculated with the quotient of absolute or toe pressure to brachial pressure. Intraarterial digital subtraction angiography and magnetic resonance angiography were performed at 1 week before and 4 weeks after each treatment and at 3 months after the latter of the 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study with undiluted nonionic contrast media (Isoview 370, Squibb Diagnostics, New Brunswick, NJ). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and foot levels were recorded with digital acquisition and hard copies in a 35 × 45–cm format. To allow a precise comparison between the angiograms that were obtained before and after the gene therapy, meticulous attention was paid to the volume of contrast used and to the timing of the image acquisition to be certain that the comparison of identical phases was possible. The diameter of the newly visible collateral vessels was documented by the comparison with a 0.9-in diameter reference wire that was taped to the skin.

Magnetic resonance angiography was performed with a 1.0-T superconducting system (Impact, Seimens, Erlanger, Germany) by means of a transmit/receive extremity coil, a body coil, or both and by means of commercially available 2-dimensional time-of-flight noncontrast-enhanced pulse sequences. The overlapping 2-dimensional time-of-flight sequences were obtained, at levels that were standardized for each patient, from the malleoli to the pelvis. The maximal intensity projections were obtained at 60-degree intervals and were used to compare the pretherapy and posttherapy studies. An increase in the number of visible vessels or an increase in the intensity or apparent size of a previously visible vessel was considered improvement.

Serum vascular endothelial growth factor levels. Enzyme-linked immunosorbent (ELISA) assays were performed at baseline and weekly for up to 12 weeks after the initial treatment to detect evidence of gene expression at the protein level. The samples were centrifuged immediately for 20 minutes at 3600 rpm at 4°C, and the serum was stored at 20°C until analysis. The serum VEGF was determined with immunoassay according to the manufacturer's instructions (R & D Systems, Minneapolis, Minn). The results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. The samples were checked with serial dilution, which was performed at least in duplicate.

RESULTS

The demographic and clinical data for the 4 men and 2 women (aged 20 to 48 years at the onset of

symptoms) are shown in Table I. All the patients had been heavy smokers for many years, and, in fact, 3 patients (4 of 7 limbs treated) had been unable to stop smoking before the initiation of the gene therapy treatment and indeed, despite counseling, continued to smoke during the study period (Table II). The 3 patients who quit did so more than 6 months before being referred to us for gene therapy. Previous medical or surgical treatment included multiple distal bypass grafts in 2 patients (all of which were occluded), prostaglandin therapy in 3 patients, and sympathectomy in 2 limbs in 1 patient. At the time of the assessment for gene therapy, necrotic lesions were present in 6 of 7 limbs. These lesions included toe gangrene in 4 limbs and the involvement of the forefoot in 2 limbs. One patient was seen with severe rest pain only. Upper extremity involvement in the form of Raynaud's phenomenon was present in 3 patients, and thrombophlebitis could be documented in 1 patient.

The average length of follow-up time at the time of this report was 14 months (range, 3 to 18 months). The intramuscular gene transfer into the ischemic limb induced minimal local discomfort for up to 72 hours after the injection. The serial creatinine phosphokinase measurements remained in the normal range. No systemic or local inflammatory reactions were noted. No antibodies to VEGF were developed in patients. To date, a careful follow-up evaluation has documented no change in visual acuity and no funduscopic evidence of diabetic retinopathy. Furthermore, no evidence of latent neoplasm has been observed. The only complication seen has been transient lower extremity edema in 3 limbs that was consistent with the VEGF enhancement of vascular permeability.[9]

Transgenic expression. The blood levels of VEGF transiently peaked between 1 and 3 weeks after the gene transfer as illustrated in the weekly serum ELISA assays (Table III). Eleven out of the total of 14 $phVEGF_{165}$ treatments (injections) were associated with an increase followed by a decrease in level of VEGF protein in the serum. Thus, although individual values vary a great deal, a typical pattern is seen in most patients after injection. The presence of peripheral edema in 3 patients occurring mainly in the treated limb also provides indirect evidence of VEGF expression and corresponded in time to the rise in serum VEGF levels.

Hemodynamic studies. Five of the 7 limbs had an ABI of less than 0.6 before the gene therapy. After the gene therapy, the ABI increased in 4 of these 5 limbs by more than 0.1 (mean increase, 0.25). The 2 limbs with ABIs of more than 0.6 had TBIs of less than 0.5, and in both instances, after the gene therapy, the TBI increased. The ABIs measured simultaneously in the contralateral limb at each time point were abnormal, which implied bilateral disease in 4 of the 7 limbs, but remained unchanged during the treatment period (Table IV). Toe plethysmography was performed where possible, and an example of the restoration of a pulsatile waveform seen 4 weeks after the gene therapy is illustrated in Fig 1. These improvements in hemodynamic pressure indices were sustained during the followup period but did not dramatically change after the second injection. Only 2 of the 6 patients were able to walk sufficiently to undergo graded treadmill testing before the gene therapy. Both of these patients had an improvement in their walking distance that was documented by follow-up treadmill testing 8 weeks after their treatment.

Angiographic studies. Digital subtraction angiography before the gene therapy documented the typical findings of Buerger's disease in all 7 limbs (Fig 2). These findings included segmental occlusive disease involving primarily the tibial and digital vessels but extending up to the distal superficial femoral

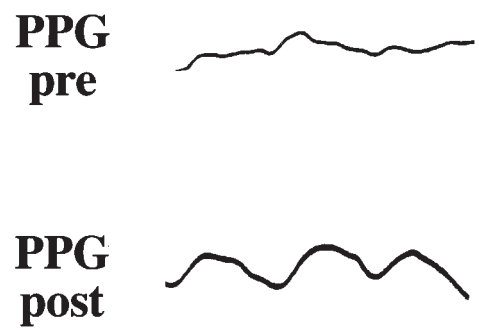

Fig 1. Toe plethysmographic tracings taken from a patient immediately before and 4 weeks after initial treatment with $VEGF_{165}$ gene therapy.

Table II. History of smoking

Patient	*Age*	*Amount of smoking (cigarettes/day)*	*Cessation of smoking*	*Change of smoking habits during Gtx*
1	33	30	yes (12 months before Gtx)	no
2	39	10	no	no
3	39	10	no	no
4	44	40	no	no
5	51	20	no	no
6	26	12	yes (5 months before Gtx)	no
7	46	30	yes (6 months before Gtx)	no

Gtx, Gene therapy.

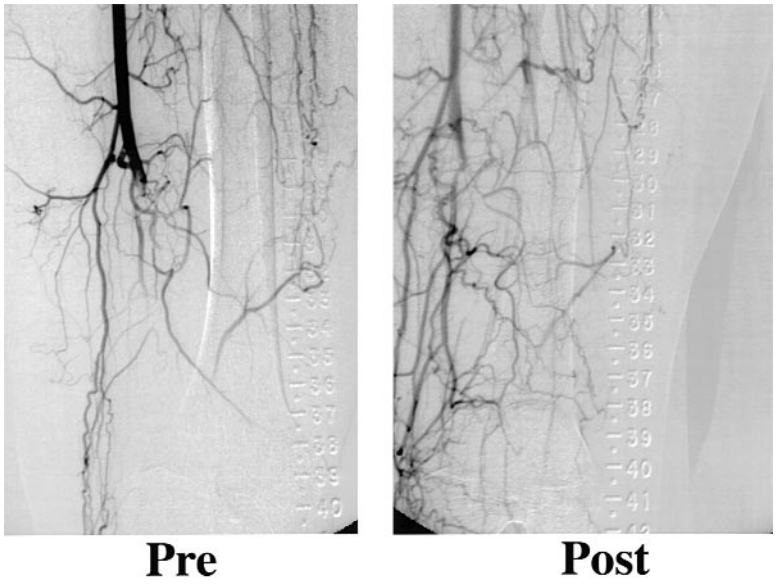

Fig 2. Digital subtraction angiogram from a patient before gene therapy showing typical findings of Buerger's disease at distal popliteal and tibial levels with normal femoral vessel proximally. Angiogram after gene therapy at 8 weeks shows significant increase in collateral formation.

Table III. Vascular endothelial growth factor serum levels

	VEFG serum levels (pg/mL)				
Patient no.	*Before first Gtx*	*At first peak*	*After first Gtx*	*At second peak*	*After second Gtx*
1	47	223	116	607	0
2	ND	59	29	888	30
3	142	24	31	37	20
4	34	668	ND	550	69
5	151	251	69	347	67
6	20	43	18	147	13
7	ND	264	53	46	47

VEFG, Vascular endothelial growth factor; *Gtx,* gene therapy; *ND,* not performed.

artery or the popliteal artery in 4 limbs. Typical corkscrew collaterals were observed, and vessels proximal to the distal superficial femoral artery were notably free of any evidence of disease. After the gene therapy, new collateral vessels that ranged in size from 200 to 800 μm in diameter were observed (Fig 2). The serial magnetic resonance angiograms of the ischemic limb showed qualitative evidence of improved distal flow in all instances, which included increased signal intensity in previously identified vessels and an increase in the number of vessels that were visible (Fig 3).

Clinical outcome. Overall, clinical improvement was observed in 5 of the 7 limbs that were treated by $VEGF_{165}$ gene therapy, whereas the 2 limbs that were seen with forefoot gangrene ultimately necessitated amputation at the below-knee level. In addition, toe amputation with limb salvage was achieved in 1 limb. The healing of toe gangrene and the resolution of rest pain was achieved in 3 limbs (Fig 4), and improved walking distance was documented in 2. In general, the clinical improvement correlated with the improvement of hemodynamic status, although 1 of the patients ultimately required below-knee amputation despite an improvement of 0.27 in the ABI. Of interest, the improvement in clinical status occurred in 2 limbs despite the documentation of failure to discontinue smoking in both of those patients.

Pathology. The histologic findings in the pathologic specimen that was obtained from 1 of the patients who underwent amputation are illustrated in Fig 5 and show the typical findings of Buerger's

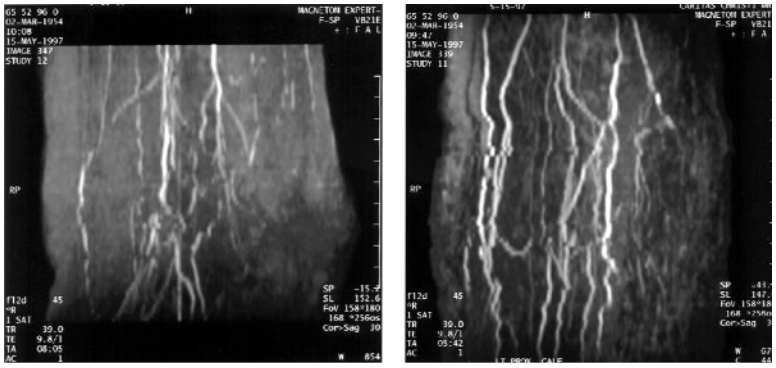

Fig 3. Magnetic resonance angiograms without intravenous contrast medium from a patient before and after gene therapy illustrating both increased number of collateral vessels and increased signal intensity in previously identified vessels.

Table IV. Ankle brachial indices of 6 patients (7 limbs)

			Ankle brachial index			
Patient no.	*Age (years)*	*Limb*	*Baseline*	*1 month after Gtx*	*2 months after Gtx*	*3 months after Gtx*
1	33	treated	0.3	0.42	0.51	0.64
		untreated	0.95	1	0.99	0.93
2	39	treated	0	0.24	0.25	0.27
		untreated	0.35	0.33	0.34	0.31
3	39	treated	0.31	0.39	0.55	0.54
		untreated	NA	NA	NA	NA
4	44	treated	0.39	0.31	0.37	0.32
		untreated	0.64	0.69	0.68	0.63
5	51	treated	0.55	0.61	0.65	0.7
		untreated	1.00+	1.0+	1.00+	1.00+
6	26	treated	0.77	0.72	0.8	0.72
		untreated	1.00+	1.0+	1.00+	1.00+
7	46	treated	0.7	0.81	0.8	0.75
		untreated	0.75	0.84	0.83	0.81

Gtx, Gene therapy; *NA,* not performed.

disease. Numerous small blood vessels—both arterial and venous—that were occluded by partially recanalized thrombus and were surrounded by an extensive inflammatory cell infiltrate are evident.

DISCUSSION

The first detailed description of a patient who was seen with the condition now known as TAO was described by Felix von Winiwarter in 1879.[10] In 1908, Leo Buerger[11] fully characterized this disease entity in a detailed pathologic study of 11 amputated limbs and, in fact, suggested the term *thromboangiitis obliterans* to distinguish it from atherosclerosis. During the first half of the 20th century, there was a tendency for the disease to be overdiagnosed, which lead to skepticism as to its actual existence.[1] At present, however, this controversy seems resolved, and there now is abundant evidence of the existence of this pecular form of occlusive disease that afflicts the peripheral arteries of young smokers.[12] Because its specificity is primarily on the basis of its clinical characteristics, the disease is perhaps best termed *Buerger's disease* rather than TOA, as suggested by Shionoya.[1]

All 6 patients who were described here were seen by us with the typical features of Buerger's disease. In each case, the age at onset was less than 50 years (mean, 32.5 years; range, 20 to 48 years), and each gave a history of heavy cigarette usage. None of the 4 males and 2 females in this study were diabetic nor

Fig 4. Photographs of feet of 2 patients showing healing of necrotic lesions of toes 6 to 8 weeks after initiation of gene therapy with phVEGF$_{165}$.

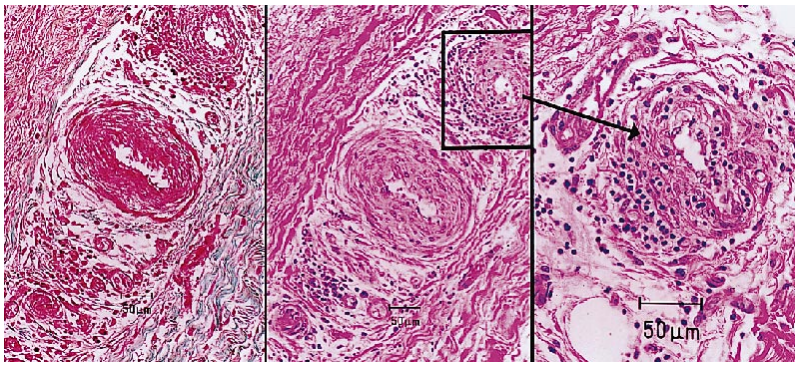

Fig 5. Representative histologic sections of tissue retrieved from a patient with Buerger's disease at time of limb amputation. Serial sections stained with elastic tissue trichrome *(left)* and hematoxylin and eosin *(middle)* show small arteries and veins containing thrombus and surrounded by significant inflammatory cell infiltrate. Note preservation of normal blood vessel architecture. Photomicrograph on *right* is a higher magnification of area indicated in middle photo.

were any seen with features of atherosclerosis obliterans. All the patients were carefully evaluated for evidence of a hypercoagulable state or autoimmune forms of vasculitis, and the laboratory investigations, which included testing for antithrombin 3, protein C, protein S, circulating lupus anticoagulants, antinuclear antibiodies, rheumatoid factors, and anticentromere antibiodies, were negative. Evidence of hypercholesterolemia and hyperhomocystinemia also were excluded. No source of systemic embolization could be documented with cardiac ultrasound scan or with abdominal computed tomographic evaluation. Upper limb involvement characterized by Raynaud's phenomenon was noted in 3 patients, and a history of superficial thrombophlebitis was present in 1.

All patients were seen with advanced critical limb ischemia with necrotic lesions present in the toes in

4 limbs and with forefoot gangrene present in 2 limbs. Only 1 patient in this series was seen with rest pain only. The patients first were seen by us an average of 7 years after the onset of their symptoms and had critical ischemia that included gangrenous lesions of the feet for an average of 5 months. Previous treatment had included multiple attempts at distal bypass grafting procedures in 2 patients, bilateral sympathectomy in 1 patient, and prostaglandin therapy in 3 patients. Three patients claimed to have stopped smoking completely more than 6 months before the referral for gene therapy, and 3 others had not been able to do so.

Thus, the patients who are described here represent a small subgroup of Buerger's disease that was first seen at a relatively late stage after having failed conservative therapy and more aggressive interventional therapy that included distal bypass graft surgery. Indeed, in the case of the first 4 limbs that were treated in this study, the patient had been advised to undergo amputation below the knee by the treating physician and had refused, and patients 5 to 7 were told they might require amputation if their limbs did not respond to conservative treatment. As such, they met the strict inclusive criteria for entry into this phase I clinical trial of intramuscular gene therapy with $phVEGF_{165}$. Indeed, patients 1 and 2 who are presented here were among the first 9 patients to enter this trial and are also included in the recently published report of Baumgartner et al.[13]

Because VEGF had not been previously administered clinically either as a recombinant protein or by means of gene transfer, this clinical trial was designed primarily to assess the safety of intramuscular gene therapy with $phVEGF_{165}$ and, in addition, to detect evidence of bioactivity in the form of both evidence of gene expression at the protein level and in terms of clinical efficacy.

From the perspective of patient safety, no significant adverse effects were observed in this group of patients. Aside from some minor discomfort at the injection sites and peripheral edema in 3 of the 7 limbs, no other side effects were observed and, specifically, there was no evidence on long-term follow-up of any systemic effects of VEGF in terms of the development of retinopathy or new tumor growth.

The analysis of gene expression at the protein level by the use of an ELISA assay for VEGF documented considerable variability from patient to patient. Although the serial VEGF levels in the individual patients tended to peak at 1 to 3 weeks after gene transfer, the degree of elevation was highly variable and did not necessarily correlate with the clinical outcome or the hemodynamic change. This may be partly because the measurements were only taken weekly, and thus, some peak values may have been missed. At any rate, the use of VEGF levels on the basis of these data remains to be clarified. Similarly, we interpret the development of peripheral edema in some patients as evidence of VEGF expression given that enhanced vascular permeability is a feature of VEGF.[14] Whereas we have documented in a previous analysis that the development of edema corresponds temporally with increased VEGF levels and is consistent with the time course of gene expression (3 to 21 days) that was established in animal studies, this finding was not a predictor of clinical improvement.[15]

The analysis of the clinical outcomes that were observed in these patients and the hemodynamic and imaging data may be cautiously interpreted as providing preliminary evidence of the potential efficacy of intramuscular VEGF gene therapy. Specifically, the ABI, or in 2 instances the TBI, increased by at least 0.1 in 4 of the 7 limbs, and, at the same time, no change was documented in the contralateral limbs. This was accompanied by the restoration of pulsatile flow in the toe pulse volume recordings as well. Clinically, rest pain was relieved and necrotic ulcers healed in all but 2 limbs—both of which ultimately went on to below-knee amputation at 3 and 7 months after gene therapy. This occurred despite an improvement in the hemodynamic studies because of advanced gangrene of the forefoot, which had been present for 4 and 5 months, respectively, before the gene therapy. The 1 patient who was seen with only rest pain not only experienced relief of the rest pain but also improvement in the claudication walking distance. Magnetic resonance angiography showed an increased flow to the calf vessels in all 7 limbs. Contrast angiography showed evidence of increased collaterals in all 7 limbs but probably underestimated the degree of collateral development; the size of the newly formed vessels in most instances is less than the resolution of standard contrast angiographic techniques.[16]

The conceptual basis for therapeutic angiogenesis with $phVEGF_{165}$ gene transfer was developed on the basis of studies from our laboratory and others. Work from several laboratories[17-20] has convincingly shown evidence of transgenic expression after the direct injection of nonviral covalently closed plasmid DNA into skeletal muscle. Whereas the transfection efficiency with naked DNA is known to be relatively low, particularly in comparison with plasmids that use viral vectors, the clinical outcomes here suggest

that the success of gene therapy is not solely a function of transfection efficiency. Because VEGF is actively secreted by intact cells, studies from our laboratory[21] have shown that meaningful biologic outcomes may be possible as a result of the paracrine effects of this secreted gene product. Furthermore, VEGF-induced angiogenesis in preclinical studies has been documented to be site specific and appears to occur predominantly at the sites of ischemia.[22] This again appears to result from the paracrine up regulation of the principle high-affinity VEGF receptor Kdr in response to factors released from hypoxic skeletal myocytes.[21] Thus, the presence of hypoxic muscle in these severely ischemic limbs may have enhanced the ability of the VEGF produced to stimulate angiogenesis in this setting. The finding of peripheral edema in the contralateral limb in some patients, on the other hand, may imply that this action of VEGF is not mediated exclusively by the same receptor.

The patients with Buerger's disease often appear with angiography to have abundant collateral vessels around the multiple distal occlusive lesions, yet the distal tissue perfusion may be extremely poor, which leads to advanced necrotic lesions as were seen in this series. Previous studies from our laboratory[23] have shown that in addition to the ability of VEGF to stimulate new collateral formation, it is also capable of improving disturbed endothelium-dependent flow in collaterally perfused limbs. It is entirely possible that the improved flow that was seen with magnetic resonance angiography in these patients was at least, in part, the result of enhanced endothelium-dependent blood flow in the already present collateral vessels.

Patients with Buerger's disease by definition tend to be relatively young and free of evidence of diffuse atherosclerosis in their native arteries. Recent studies from our laboratory have shown that collateral vessel development is impaired as a function of age,[24] hypercholesterolemia,[25] and diabetes.[26] Thus, this group of patients who are younger and nondiabetic with normal cholesterol levels may indeed represent the ideal candidates for angiogenic therapy.

In summary, this report documents the results in 6 patients who met the clinical criteria for Buerger's disease who were treated with intramuscular phVEGF$_{165}$ gene therapy as a part of a larger clinical trial of this therapy in patients with advanced peripheral vascular disease who could not be treated surgically or percutaneously. These preliminary results document augmented perfusion in all the limbs treated that was sufficient to bring about the healing of gangrenous ulcers or toes in 4 limbs and to relieve the rest pain in all but 2. The requirement for amputation in the 2 limbs that were seen with already established necrotic lesions of the forefoot suggests that gene therapy, to be successful, must be instituted earlier in such cases. The therapy for patients with Buerger's disease who are seen with advanced critical limb ischemia has generally been futile with a high risk of amputation, especially in those patients who continue to smoke. Fiessinger and Schafer,[27] however, reported good results in such patients with 6-hour daily infusions of Iloprost (a prostaglandin analog; Berlex, Montville, NJ) given over a 28-day period. Aside from the complexity and cost of such a treatment protocol, however, Iloprost is not currently available in the United States.

Although the data that were presented here are preliminary and nonrandomized, we believe the data support the further investigation of therapeutic angiogenesis in patients with Buerger's disease who are seen with critical limb ischemia that is unresponsive to other therapeutic methods.

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DISCUSSION

Dr Anton N. Sidawy (Washington, DC). Vascular endothelial growth factor, VEGF, is a peptide growth factor that causes endothelial cell proliferation. VEGF has been found to promote the growth of new blood vessels in the myocardium and peripheral circulation. VEGF exerts its effects via 2 types of receptors located on the vascular endothelium and is found in various isomers that defer in their isoelectric charge and their affinity to heparin. VEGF DNA can be introduced either by using an adenovirus vector or by injecting a naked plasmid DNA, encoding the 165 amino acid VEGF isoform. Both have been shown to produce formation of new blood vessels experimentally.

Dr Symes and his colleagues ought to be congratulated on being in the forefront in studying this important and promising molecule experimentally and clinically. He reported on the treatment of 6 patients with Buerger's disease. As you all know, this disease is difficult to manage in its late stages. The authors report encouraging results in limb salvage with VEGF in these patients. I have the following questions for Dr Symes.

Would you expand on the difference between introducing the naked DNA and introducing the DNA by means of adenovirus vector? I have heard your group reporting on injecting naked DNA and I was intrigued by the results.

Did you perform any evaluation to detect tumors, especially cancers, that the patients may have had at the time of treatment? Angiogenesis is a process used by tumors to gain blood supply from surrounding tissues so that they will grow. Are you helping that process by injecting VEGF DNA?

As we know from randomized trials, when treating patients with foot ulcers, about 30% or more of the placebo group patients have ulcers that heal. This is probably as a result of the extra care they receive while in the study. Your study is a nonrandomized safety study, so there was no control group for comparison. Would you comment on the possible bias involved and whether the results you have obtained are as a result of the extra care that these patients were given?

Also, please comment on smoking in these patients. In your study, 3 of 6 patients stopped smoking. Are the patients who ended up with amputations the ones who did not stop smoking?

You stated that you injected the material in both limbs

in patients with bilateral disease. Why is this necessary because DNA is causing increased VEGF levels in the blood and should influence both extremities? Why must the material be injected directly in the affected limb?

I would like to thank the Society for the privilege of discussing this paper.

Dr James F. Symes. Thank you for your kind comments and for your insightful questions.

First, with regard to our use of naked DNA versus that incorporating an adenoviral vector, it is well documented experimentally that incorporation of an adenoviral vector into any plasmid used for gene therapy will enhance its transfection efficiency.

On the other hand, adenoviral vectors may have exposed patients to side effects related to the use of the viruses themselves. So, it was primarily from the point of view of safety that we chose to use naked DNA rather than DNA with a viral vector.

Furthermore, as I indicated, we believe that certain features of VEGF allow it to be biologically effective, even though we used naked DNA. However, if one can show that adenoviral vectors are indeed safe, I expect that most studies done with gene transfer will probably incorporate them.

As far as looking for tumors, all of our patients were meticulously screened with head, thorax, and abdominal computed tomographic scans before being admitted into the study.

We also have looked for evidence of tumor appearance after surgery and, at least to this point, have not seen any evidence that the VEGF has caused problems in this regard. Further, we think one of the potential advantages of using gene therapy as opposed to the administration of the protein is that you get a relatively low systemic level of the protein with gene therapy but for a longer period of time (2 or 3 weeks), and hopefully that might be safer than if you administered the protein intravenously or intramuscularly at higher dose levels.

Your question about spontaneous healing of the ulcers is a good one. It would require a randomized study to be absolutely certain that some of these ulcers would not have healed if the patient had received some sort of placebo. However, I can tell you that, at least up to the point that these patients were seen by us, all attempts by the physicians treating them before our gene therapy trial had been unsuccessful in healing these ulcers. In addition, the majority of patients who do not respond to gene therapy end up with an amputation.

Of the 3 patients who did not stop smoking, 1 did indeed go on to below-knee amputation, 1 presented with rest pain only and his rest pain was relieved by gene therapy despite the fact that he did not stop smoking, and the third patient healed his toe ulcer. Thus, 2 of the 3 people who kept on smoking, albeit at a reduced rate, did improve with gene therapy.

With regard to bilateral disease, we did not treat the 2 limbs in that patient at the same time. She was seen initially with advanced gangrene of 1 forefoot, received gene therapy for that limb, but ended up with a BK amputation. She is one of the patients who did not stop smoking. Within about 2 or 3 months, she had necrotic toes on her other foot, which were then treated with gene therapy successfully.

I thank you again for your comments.

Dr Peter J. Pappas (Newark, NJ). In working with DNA and RNA, one of the things I have learned is that if you breathe on these substances, they degrade. What I do not understand is how naked plasmid DNA injected into the muscle can somehow evade the body's own DNAases and RNAases? What is it about this intramuscular injection that allows it to incorporate into the muscle's own nuclear structure and yet evade breakdown?

Dr Symes. We have looked at the pathologic specimens on a couple of patients who have had amputations. We have seen evidence of broken bits of plasmid in the tissues in and around the injection sites, but presumably enough of the DNA does get incorporated into the cells to produce a meaningful biologic effect.

Dr Pappas. It is just difficult for me to comprehend this, because even in the animal studies with adenoviral vectors, where you can look at protein expression and gene expression, you only get a response for up to 3 weeks and then the body attacks the vector and treats it like a foreign substance. I just cannot comprehend how this works, if it does work.

Dr Symes. Our studies also indicate VEGF production for up to 3 weeks, and it is for that reason that we repeat the gene transfection, administering a second dose 4 weeks after the first.

Dr Frank J. Veith (Bronx, NY). I think many of us are skeptical. Even though 1 of the patients you showed was our patient, and I agree it was a dramatic response, I think that before we are going to accept the hypothesis as proven, we would like to see some kind of controlled study. All of us have had the experience of seeing a patient who has had multiple previous failures with extensive ulceration and gangrene and who refuses amputation, and, lo and behold, after 6 months they come back healed. Maybe it occurs for other reasons. Thus, before we accept this, there should be a randomized, prospective study. I wonder if you are planning such a study or at least some kind of controlled study?

Dr Symes. I totally agree with you, and frankly, I was probably as skeptical as anyone that there would be any benefit when this study was started. In the laboratory, we can get dramatic improvement in rabbits and rats and impressive evidence of stimulation of angiogenesis. However, these patients are end-stage. The thought that putting a little DNA into these legs would produce a beneficial effect is good reason for skepticism.

I agree that to prove the therapy you need a randomized trial as you do for any new therapy, and ultimately this will need to be done.

At this point, I think we still need to refine the therapy to the point where we are absolutely convinced that we are seeing a strong biologic effect under all circumstances. The purpose of this phase I trial was really to try to bring a new

therapeutic concept to the clinic. It will be a few years before this is something that will be an alternative in the routine treatment of patients with peripheral vascular disease.

Dr K. Craig Kent (New York, NY). I share Dr Veith's skepticism. It is certainly going to take time before we sort out all of this. You noticed that there was an increase in the ankle brachial indices of 0.1 in these patients. What do you think the error of that test is? Do you really think that 0.1 is a significant increase in circulation in these patients?

You noticed a great deal of edema. In some of these patients were you worried about a compartment syndrome? Is this something that we need to consider?

And my third question is, how did you decide where to do the injections? Were they truly random, or did you have some pattern? There is a great deal of muscle in the thigh and the calf, and it would seem that you would have to have had some pattern of injection to be able to connect the collaterals.

Dr Symes. Unfortunately, the ankle brachial index is not an effective way to show increased angiogenesis at the microvascular level. I can tell you that in conducting the trial, we have attempted to carry out these noninvasive studies as meticulously as possible. We document it twice before starting the therapy. I think any change that you see is as real as it can be. If you use the standard criteria that a change of greater than 0.1 or 0.15 implies some kind of significant change in the circulation, then in some of these patients we have seen increases of 0.2 or 0.3. However, I think it is a gross way to measure what is going on here and probably is not going to show us a great deal.

The edema was a concern. Compartment syndrome was not seen in any of these patients. We did treat them fairly aggressively when we had the opportunity to detect the edema early. Once we recognized it in a couple of the early patients, the patients were told that if leg swelling developed they were to get back to us right away and they were put on diuretics. It is transient and goes away within 1 to 2 weeks.

In terms of where we inject the gene, our concept was 2-fold. First, we had to inject it into viable muscle. Second, hypoxic muscle might be a better site for VEGF to exert its effect because of the upregulation of the receptors in that setting. So, we have tended, as the study has gone along, to try to administer it further distally in the limb and even have tried to administer it down near the foot level in a couple of patients.

Dr Theresa Jacob (Brooklyn, NY). I enjoyed your presentation, and I have a few questions for you.

My first concern relates to your bioreactivity studies. Was the VEGF gene or its product detected at 8 weeks? Were long-term time course experiments for VEGF gene expression performed? Because you have used the transfer of naked DNA, we would like to know the duration of this expression in the vascular tissues. At Maimonides Medical Center, we studied the bioreactivity of the p53 gene in a rat carotid injury model, and we observed a limited life span of this transfected gene. However, its expression could be prolonged by the use of selective immunosuppression with CTLA4Ig.

The other question is regarding the amputated limb. Did you do any in situ hybridization tests or immunohistochemical studies on that amputated limb? What were the results? Could you detect the VEGF DNA sequence or localize its expression in any of the tissues?

Dr Symes. Actually the answers to both questions are similar. Our studies in the laboratory and in the patients that measured the VEGF protein levels have documented that most of the gene that is produced is produced between 1 and 3 weeks and that when you get out to 4 weeks the gene is no longer producing protein. The one amputation specimen was obtained from an amputation that occurred about 3 months after the gene injection, so we were not able to document any evidence of protein expression at that time.

Dr Michael A. Golden (Philadelphia, Pa). I enjoyed your presentation and feel that there is great promise with this. I have just a few questions for you.

You had mentioned earlier in answer to someone's question that you saw some DNA. Was that in one of your animal models where you biopsied the muscle, or was it an amputated limb? Is there any evidence of muscle necrosis or inflammatory response? What is the size of the material that you are injecting? Obviously, you have a promoter/enhancer construct to drive expression. You should be able to identify that if you looked for it. Have you looked for it?

Dr Symes. Yes, and indeed we have seen it. We use a cytomegalo-virus promoter/enhancer. The pieces of plasmid that I mentioned we saw were in the amputated specimen near the sites of injection of the DNA. There was no evidence of any muscle necrosis. We did serial creatine phosphokinase measurements on these patients and found no increase in creatine phosphokinase. The patients noticed some mild discomfort near the injection sites in a few instances, but there was no gross inflammatory response evident.

Dr Golden. Was there pulmonary edema or any problem with edema elsewhere?

Dr Symes. No.

Dr Golden. So, you would find edema in the contralateral leg but in no other organs?

Dr Symes. We did see a little edema in the contralateral leg on occasion, but that is the only other place we saw it.

Dr Maciej Dryjski (Buffalo, NY). Have you seen any difference in the VEGF level among the patients who fail therapy and among those who improved?

Dr Symes. No. The transient increase in VEGF levels is highly variable. The manuscript includes a table that shows all of the individual measurements. You will note that there is a great deal of variability from patient to patient. The only pattern that we saw was this biphasic peak that occurred 1 to 3 weeks after each injection. I think that is partly because of the timing of when the sample was taken (once per week). So, one could miss the peak value in 1 patient and hit it in another, but there certainly was a good deal of variability between patients.